Evaluation of arbitrarily primed polymerase chain reaction analysis for typing *Legionella pneumophila*

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Objective: To evaluate the performance of arbitrarily primed polymerase chain reaction (AP-PCR) analysis in epidemiologic typing of *Legionella pneumophila*.

Methods: Sixty-two isolates of *L. pneumophila* of serogroups 1, 3, 6 and 10, including epidemiologically related and unrelated isolates, were analyzed by AP-PCR using the primer BG2. Twenty-six of the serogroup 1 isolates were typed by pulsed-field gel electrophoresis (PFGE).

Results: AP-PCR analysis showed 98% typeability and complete reproducibility. A majority of unrelated isolates of each serogroup could be distinguished (discrimination index: 92%). Clinical isolates showed AP-PCR patterns indistinguishable from those of the isolates of the related environmental source. PFGE and AP-PCR results were in agreement for 88% of isolates.

Conclusions: Single-primer AP-PCR analysis can be used as a simple and reproducible screening method for typing *L. pneumophila* strains of different serogroups.

Key words: *Legionella pneumophila*, bacterial typing, AP-PCR, PFGE

INTRODUCTION

Among the genus *Legionella*, *L. pneumophila* is the species that most commonly causes pneumonia, particularly in immunocompromised patients. Transmission of *Legionella* from environmental sources to susceptible patients can occur by inhalation of aerosols from contaminated water [1–3], or by contact with air-conditioning systems.

To control nosocomial legionellosis, measures must be taken to reduce the contamination level of *Legionella* in hospital water systems or to prevent exposure of susceptible patients to contaminated water sources. Rapid typing methods are useful to trace the source of infection. Many methods have been used for typing *Legionella*, including serotyping, monoclonal antibody (Mab) subgrouping [4], plasmid analysis [5], restriction fragment length polymorphism (RFLP) analysis [6–8], including by pulsed-field gel electrophoresis (PFGE) [8–10]; ribotyping [11], multilocus enzyme electrophoretic (MEE) typing [5,8], repetitive element PCR analysis (rep-PCR) [12], arbitrarily primed PCR (AP-PCR) analysis [10,13,14], and, more recently, amplified fragment length polymorphism (AFLP) [15]. Molecular typing of *L. pneumophila* serogroup 1 strains by PCR amplification of variable genomic regions with arbitrary sequence primers, such as BG2, provides a discrimination similar to that of PFGE of genomic macrorestriction fragments [16]. The reliability of PCR fingerprinting using the single primer BG2 has been reported in comparison with other molecular typing methods [16]. However, in that study reproducibility was not systematically investigated. The reproducibility of AP-PCR analysis is affected by a number of experimental parameters [17]. The poor

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reproducibility of this method results mainly from the low-stringency conditions under which primer–template annealing occurs [18]. In this study, the typeability, reproducibility and discriminatory power of single-primer AP-PCR for typing *L. pneumophila* of various serogroups (serogroups 1, 3, 6 and 10) were further evaluated [19].

MATERIALS AND METHODS

Bacterial strains

Two collections of strains were selected for this study on the basis of diversity of epidemiologic sources and inclusion of sets of outbreak-related strains from patients and from sources of contamination in the environment. A first set of strains included 36 strains of *L. pneumophila* of four serogroups (serogroups 1, 3, 6 and 10) recovered from unrelated settings in Belgium (n=6) and in Hong Kong (n=1) between 1981 and 1990. These strains included clinical isolates from cases of legionellosis (n=16), environmental isolates from water distribution systems (n=19), and air-conditioning systems (n=1).

A second set of *L. pneumophila* serogroup 1 strains included reference strain *L. pneumophila* NCTC 11404, clinical isolates from two hospitals in Brussels, the Erasme Hospital (n=9) and Hospital B (n=1), clinical isolates from community-acquired cases (n=9) and isolates from Erasme Hospital water (n=6) recovered between 1988 and 1994. These strains were cultured on BCYE agar (Oxoid, Unipath, Ghent, Belgium) for 48 h at 37 °C, identified by direct immunofluorescence and typed by PFGE after macrorestriction with endonuclease *Not*I as previously described [8].

Serotyping

Colonies from culture on BCYE agar (48 h) were formalinized and examined by direct immunofluorescence with a fluorescein-labeled rabbit antiserum (SCIMEDX, Denville Scientific, Denville, NJ).

AP-PCR typing

Genomic DNA was extracted from 72-h-old cultures on buffered charcoal yeast extract (BCYE) agar, by using the guanidium isothiocyanate extraction method as previously described [1] followed by one step of chloroform/isoamyl alcohol treatment. DNA concentration was adjusted to approximately 25 ng/μL by comparison with samples containing a known amount of lambda DNA (Life Technologies, Merelbeke, Belgium) as estimated visually by electrophoresis in 1% agarose gels after staining with ethidium bromide.

Duplicate typing was performed on DNA from each of the 62 strains in two PCR experiments. The reaction used 100 μL of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 200 μM each of the deoxyribonucleotide triphosphates, 100 pmol of primer BG2 (5′-TACATTCGAGGACCCCTAAGTG), 25 ng of DNA template and 2.5 U of *Taq* polymerase (Ampli Taq, Perkin-Elmer Cetus Corp., Norwalk, Conn.) in a Biomed model 60 thermocycler (Technolab, Alkmaar, The Netherlands). PCR conditions consisted of an initial denaturation step of 5 min at 94 °C followed by 45 cycles of consecutive denaturation, annealing and DNA chain extension (30 s at 94 °C, 1 min at 30 °C, 2 min at 74 °C), ending with a final extension step (5 min at 74 °C). The AP-PCR products were separated by electrophoresis in 2% agarose gels and visualized by ethidium bromide staining. A 1-kb ladder (Gibco BRL, Merelbeke, Belgium) was used as molecular size marker. Products of duplicate PCR assays were analyzed on the same gel. The interpretation of DNA fingerprints was carried out by visual examination, taking into account both major and minor DNA bands. Identical patterns were assigned to the same DNA type, each designated by a letter. Highly similar patterns differing by one DNA fragment were assigned to minor subtypes of the same major type, each subtype being characterized by a numerical suffix added to the letter code.

Macrorestriction analysis of genomic DNA by PFGE

This was performed as previously described [8]. Restriction fragments were separated by PFGE through a 1.5% agarose gel in 0.5 Tris–borate–EDTA (TBE) buffer run at 200 V. The interpretation of DNA fingerprints was carried out by visual examination. The criteria of interpretation of the PFGE patterns proposed by Tenover et al [20] are not applicable in this case, because *Not*I PFGE patterns of *Legionella* include less than 10 distinct fragments. Therefore, following the previous study [8], patterns that differed by more than a single fragment were assigned to different major types.

RESULTS AND DISCUSSION

For optimization of the method, PCR analysis with use of the previously selected arbitrary primer BG2 at different annealing temperatures (25 °C, 30 °C and 40 °C) was first evaluated on a limited number of strains. For the evaluation of reproducibility, PCR at an annealing temperature of 30 °C was selected, as it produced more informative AP-PCR patterns. Moreover, it has been proposed that the rate of accumulation of PCR products during amplification results from a balance between the synthesis of DNA and degradation of the DNA which results from the

elevated denaturation temperature. It has been shown that when the denaturation time was reduced from 1 min to 30 s, the yield of PCR products was increased approximately 10-fold [21]. Therefore, we used a denaturation step of 30 s. It has also been shown that AP-PCR analysis requires larger amounts of *Taq* polymerase than PCR amplification with use of primers that hybridize completely with target sequences [22]. On the other hand, the use of excess concentration of enzyme also affected AP-PCR products, probably as a result of increased concentration of some inhibitors. An increase in the concentration of polymerase from 1.25 U to 2.5 U per 100 μL, led to AP-PCR profiles that were more easily interpretable.

AP-PCR typing with primer BG2 generated arrays of 2–17 DNA amplimers ranging in size between 350 bp and 2000 bp. AP-PCR analysis of the 62 strains resulted in 21 major patterns and two subtypes. Typeability was 98%. If PCR profiles with ≥2 bands differences are considered as distinct major types, duplicate PCR analysis of the DNA extracts gave 100% reproducible types (Figure 1). Minor variation of a single band occurred in 2 of 62 duplicate PCR patterns. If strains of distinct sources are considered, the discriminatory power of AP-PCR was 92%, close to the level of 95% recently recommended [19]. We selected the single primer BG2 because we found it at least as discriminating as the primer combination BG2–ERIC2 for typing *L. pneumophila* serogroup 1 [16]. Even if the discriminatory power of AP-PCR could be marginally increased by using additional primers, the use of two different primers does not always expand it, as shown by Grattard et al [14]. In a recent study [23], a combination of primers was carefully selected from a larger set based on testing their discriminatory ability on a few isolates of *Legionella*.

Table 1 shows strains (n=62) isolated during the period 1981–94 from environmental sources and nosocomial cases of legionellosis acquired in four hospitals, one nursing home (Courcelles) and a chemical plant (Seneffe), 11 community-acquired cases, and an air-conditioning system, and reference strain NCTC 11404. Each of the four serogroups could be discriminated by using AP-PCR analysis with BG2 as a single primer. Concordance of AP-PCR typing with PFGE analysis was 88% (23/26 strains). These results indicate improved discrimination in comparison with a previous study [10], in which a number of unrelated clinical and environmental isolates were assigned to the same AP-PCR type even though they were discriminated by PFGE. In the majority of cases, strains of the same serogroup that were isolated from distinct sources were subdivided into different PCR types. Some exceptions also occurred, including: three serogroup 3 isolates with pattern P recovered from water samples from Seneffe and Courcelles, two cities located in the Hainaut Province in Southern Belgium; three serogroup 1 isolates with pattern A from sporadic community cases living in various parts of the country; and two serogroup 1 isolates with pattern D1 from unrelated cases acquired in Belgium and Turkey.

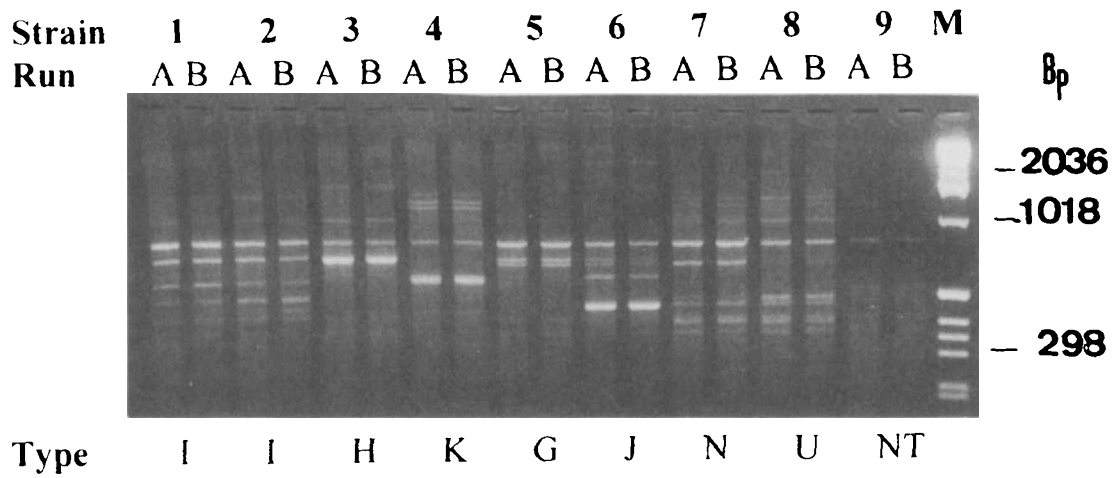

Figure 1 Run-to-run (A–B) reproducibility of AP-PCR with use of the single primer (BG2). Patterns of nine strains of *L. pneumophila* belonging to serogroups 1 and 10 isolated from Hospital A. Lanes 1–6: *L. pneumophila* serogroup 1. Lanes 7–9: *L. pneumophila* serogroup 10. M, DNA molecular size marker, 1-kb ladder.

Table 1 AP-PCR patterns of clinical (n=35) and environmental isolates (n=27) of *L. pneumophila* of serogroups 1, 3, 6 and 10, in comparison with PFGE patterns for a subset of serogroup 1 isolates

Sero-group	Epidemiologic category	Source of isolation[a]	Year of isolation	No. of isolates	AP-PCR type	PFGE type
1	Nosocomial cases	Erasme Hospital, Brussels	1990–94	2	A	Ia
			1991–94	6	A	Ib
			1994	1	E	XV
	Water system	Erasme Hospital, Brussels	1994	6	A	Ib
	Nosocomial cases	Hospital. A, Brussels	1981–85	3	G, H, I	ND
	Hot water system	Hospital A, Brussels	1984	1	I	ND
	Community-acquired cases	Lasne Chapelle St Lambert	1990	1	A	Ib
		Brussels	1992	1	A	Ib
		Namur	1992	1	A	Ib
		Brussels	1990	1	B	Ib
		De Haan–Wenduine	1992	1	C	XIII
		De Haan–Wenduine	1992	1	C	XIII
		Turkey	1992	1	D1	XII
		Brussels	1988	1	D1	II
		Mons	1994	1	E	XV
		Brussels	1987	1	K	ND
	Nosocomial case	Hospital B, Brussels	1989	1	D2	VII
	Reference NCTC 11404 Clinical isolate	USA	1981	1	F	XI
	Travel-acquired case	Hong Kong	1984	1	J	ND
	Shower-bath	Chemical plant, Seneffe	1990	3	L, L, M	ND
3	Nosocomial case	Hospital A, Brussels	1985	1	O2	ND
	Shower-bath	Chemical plant, Seneffe	1990	1	P	ND
	Hot water	Nursing home, Courcelles	1988	2	P	ND
6	Nosocomial cases	Hospital A, Brussels	1981–87	8	Q	ND
	Hot water system	Hospital A, Brussels	1984	1	Q	ND
	Shower-bath	Chemical plant Seneffe	1990	5	R	ND
	Hot water	Nursing home, Courcelles	1988	3	S, S, O1	ND
	Hot water	Hospital C, Mons	1988	3	T	ND
10	Nosocomial cases	Hospital A, Brussels	1983	2	NT, U	ND
	Air-conditioning system	Public building, Brussels	1987	1	N	ND

NT=non-typeable; ND=not typed.
[a]All cities are located in Belgium unless otherwise specified.

Sets of matched serogroup 1 and 6 clinical isolates and isolates from the source of contamination in hospital A showed the same AP-PCR types (types I and Q respectively). All isolates from nosocomial cases in Erasme hospital were assigned to the same major type (A/I) by AP-PCR and PFGE (*Not*I), except for one strain with a different pattern (E/XV). These nosocomial strains were indistinguishable from strains (n=6) isolated from the hospital water system (Figure 2). The source infection with type E/XV was not found, as this clone has not been identified in the Erasme hospital water system. Among serogroup 1 clinical isolates (n=16) from sporadic community-acquired cases of legionellosis, 13 strains were distinct from the endemic nosocomial type described above by PFGE and/or AP-PCR typing. Two strains exhibiting the same AP-PCR type (D1) were different by PFGE analysis. Four strains from community-acquired cases from Belgium were assigned to the PFGE type *I* found in nosocomial case strains from Erasme hospital, suggesting that this type may be present in other environments. Three of these strains also showed the same AP-PCR pattern, whereas the fourth strain's pattern lacked one small fragment and showed an additional fragment of larger size, and it was therefore categorized as a distinct major type. However, a single genetic event could have introduced a new primer binding site in the genome of this strain, resulting in this two-band difference from the ancestral DNA pattern. Therefore, in this particular case, the criterion of a two-band difference applied to assign strains to different major AP-PCR types appears to be too stringent.

Based on excellent concordance with ribotyping and RFLP analysis, the use of the more complex PCR-based method of AFLP analysis appears very promising for typing *Legionella* [15]. However, the good reproducibility and discrimination achieved here by using

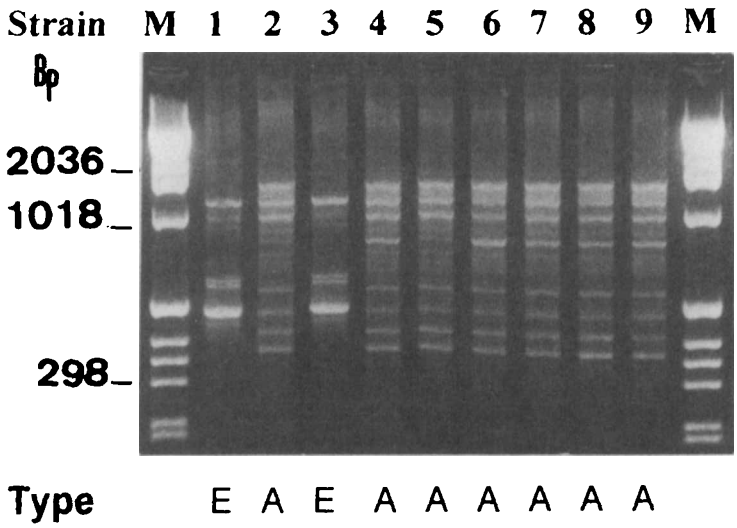

Figure 2 AP-PCR (BG2) patterns of strains of *L. pneumophila* serogroup 1 isolated from Erasme Hospital. Lanes 1–3: clinical isolates. Lanes 4–9: isolates from water distribution devices. M, DNA molecular size marker, 1-kb DNA ladder.

AP-PCR analysis with the single primer BG2 makes it a simple, rapid and cost-effective screening method for typing *L. pneumophila* strains of different serogroups. Confirmation of results by a second method is advisable given the limits of the method, as currently proposed for other molecular typing systems [19].

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